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Fluorescent Formazans and Tetrazolium Salts – Towards Fluorescent Cytotoxicity Assays

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Abstract: Formazan-based colorimetric cytotoxicity assays, such as the MTT assay, are typically used to assess cell viability with only metabolically active cells reducing tetrazolium salts into the formazans, which is then quantified by absorbance. Fluorescence offers several advantages compared to colorimetric assays and would enable techniques such as flow cytometry and confocal microscopy to be used for analysis. Here, fluorescent formazans 10, 11 and 12, and their corresponding tetrazolium salts 13, 16 and 24, respectively, were synthesised by incorporation of a known fluorophore backbone (coumarin, fluorescein and rhodol) with disruption of the conjugated system preventing or reducing fluorescence of the tetrazolium salts. The tetrazolium moiety was able to quench the fluorescence of the incorporated fluorescein and O-methyl rhodol, whereas with the coumarin-based tetrazolium salt the fluorescence was only quenched under acidic conditions. These tetrazolium salts were successfully reduced to the fluorescent formazans with cells and offer a step forward in the development of fluorescent cytotoxicity assays.

Keywords: Fluorescence, MTT, cytotoxicity assay, formazan, tetrazolium salt, synthesis

1. INTRODUCTION

Formazans were first described at the end of the 19th century but were largely ignored until the 1940's when their potential as localisation stains in living systems was reported [1,2]. Interest in formazans increased after the discovery that the addition of triphenyltetrazolium chloride **1** to cells resulted in reduction of the pale yellow **1** to the deeply coloured triphenylformazan **2** (Figure 1A) [3]. This discovery resulted in the development of the formazan-based colorimetric cytotoxicity assay (typically called an MTT assay) using 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) **3** with the dye only reduced by cells that are metabolically active to give 3-(4,5-dimethylthiazoyl-2-yl)-2,5-diphenylformazan **4** (Figure 1A) [4]. The mechanism of MTT reduction is still debated, but it is clear that it is reduced intracellularly. MTT was originally thought to be up-taken by endocytosis and reduced in perinuclear vesicles, such as lysosomes and endosomes [5]; however, MTT has since been used to measure membrane potentials, which is only possible if the tetrazolium salt can permeate the cell membrane [6]. Inside cells, the tetrazolium salt **3** is believed to be reduced (in an NADH/NADPH dependent manner) to give the strongly coloured formazan **4**,

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which is readily quantified by absorbance and can be used as a cellular viability marker [7]. However, the reduction product **4** is not soluble in aqueous media, requiring an solubilisation process, which complicates the assay and introduces error.

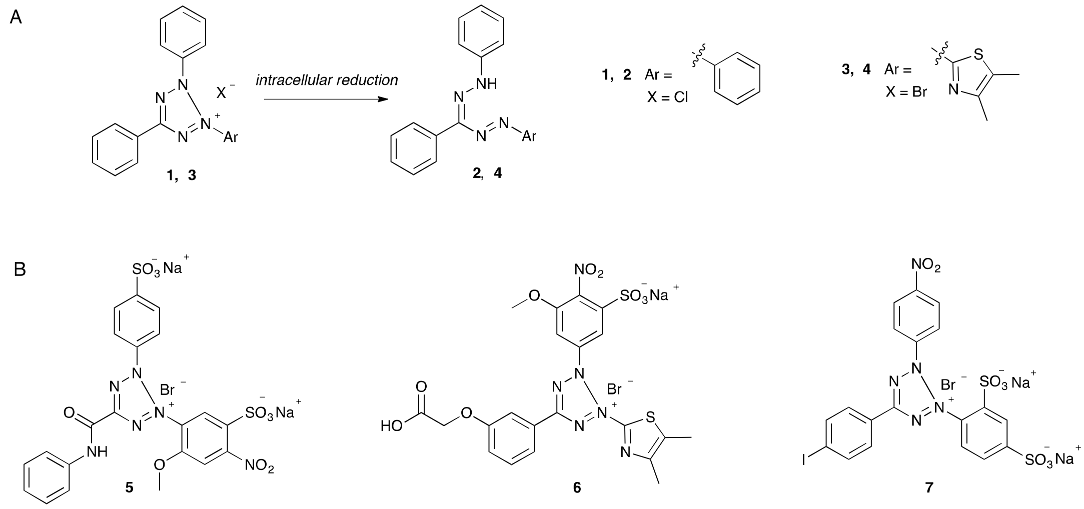


Figure 1. (A) Triphenyltetrazolium chloride **1** and MTT **3** are reduced *in vitro* to coloured formazans **2** and **4**, respectively. (B) Tetrazolium salts **5**, **6**, and **7** used in the 2nd generation cytotoxicity assays, which result in the formation of water-soluble formazans.

Sulphonated tetrazolium salts, including 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide **5** and 3-(4-5-dimethylthiazoyl-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium salt **6**, were developed to increase the aqueous solubility of the formazans, eliminating the need for a solubilisation step in the assay (Figure 1B) [8]. However, their cellular uptake is limited and the reduction is not as efficient as for MTT **2** and requires the use of an intermediate electron acceptor (such as phenazine methosulfate) to promote reduction [9, 10]. Based on these, another class of water soluble tetrazolium salts (WSTs) was developed, including 4-(3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio)-1,3-benzene disulfonate salt (WST-1) **7** (Figure 1B) [11], and other WSTs similar to **7** have become available for use in cytotoxicity assays. Although the tetrazolium salt is reduced to a highly water-soluble formazan, the mechanism of reduction is significantly different to the reduction of MTT, with reduction by membrane based enzymes at the cell surface.. Additionally, these WSTs may be reduced by other species in the media such as glutathione [12].

Fluorescence offers several advantages compared to colorimetric assays, such as sensitivity. Fluorescent cytotoxicity assay would enable techniques such as flow cytometry and confocal microscopy to be used for analysis. The most frequently used fluorescent assay is the Alamar blue (or resazurin) assay [13], which has been used to test for bacterial contamination in milk since the 1950’s. In the Alamar blue assay, resazurin is reduced by oxidoreductases to generate the strongly fluorescent resorufin ($\lambda_{Ex}/\lambda_{Em}$ 570/590 nm); however, resorufin can be further reduced to a non-fluorescent dihydroresorufin, which in cytotoxicity assays can significantly underestimate cell viability. Given the utility of the MTT assay and the benefits of fluorescence-based detection it is surprising that there is not currently a fluorescent formazan-based cytotoxicity assay for mammalian cells. 3-Cyano-1,5-ditolyl tetrazolium chloride **8**, which upon reduction gives a fluorescent formazan **9** ($\lambda_{Ex}/\lambda_{Em}$ 488/630 nm) (Figure 2), and has been used as a viability assay for anaerobic bacteria [14] but cannot be used with eukaryotic cells as it cannot cross the eukaryotic cell membrane [15]. Although **9** is commonly used as an indicator of bacterial viability, several studies have raised questions about the accuracy of the assay as different species give different responses and **8** may also be reduced by chemical additives in the culture media [16, 17].

Here, we present the design and synthesis of fluorescent formazans and their corresponding tetrazolium salts.

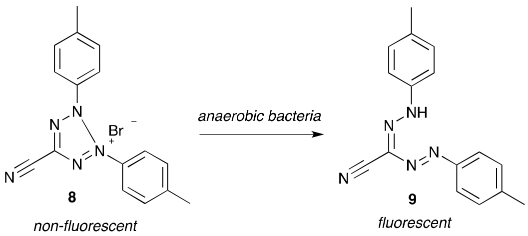


Figure 2. Non-fluorescent 3-cyano-1,5-ditolyl tetrazolium chloride **8** is reduced by anaerobic bacteria into fluorescent 3-cyano-1,5-ditolylformazan **9** ($\lambda_{Ex}/\lambda_{Em}$ 488/630 nm) [14]. The two methyl groups in the phenyl rings of **9** are essential for the fluorescence as the structurally similar 3-cyano-1,5-diphenylformazan is non-fluorescent.

2. RESULTS AND DISCUSSION

It is difficult to predict whether or not a molecule will be fluorescent; however, planarity and extended conjugation are common features in fluorescent molecules [18], but not all highly conjugated molecules are fluorescent (for example MTT **3** and the corresponding formazan **4** are not fluorescent). Here, we targeted fluorescent formazans using three known fluorescent cores (coumarin, fluorescein and rhodol). The hypothesis was that a “switch-on” fluorescent assay, similar to the MTT assay, would be possible by introducing a fluorophore into the formazan, with disruption of the conjugated system preventing or reducing fluorescence, *i.e.*, the positive charge of the corresponding tetrazolium salt (Figure 3).

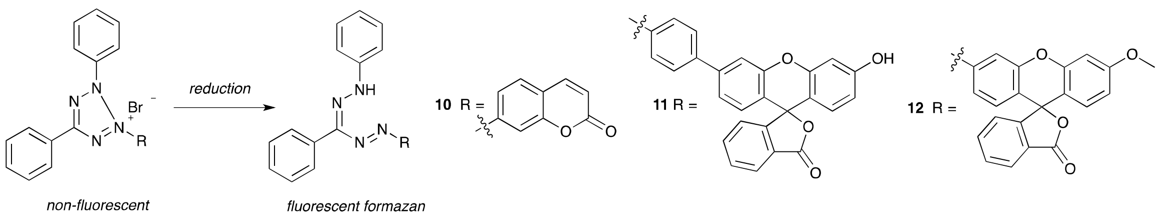
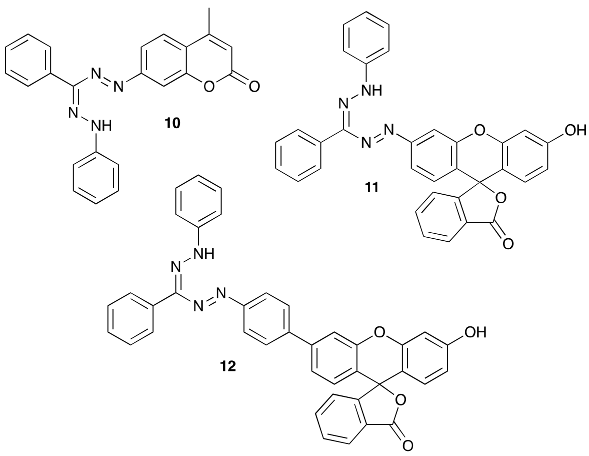


Figure 3. Design of fluorescent formazans **10**, **11** and **12**, incorporating coumarin, fluorescein or rhodol-based fluorophores, for cytotoxicity assays. The fluorescence was postulated to be quenched ("turned-off") in the corresponding tetrazolium salts, proving a "switch-on" mechanism for the fluorescence detection upon cellular reduction.



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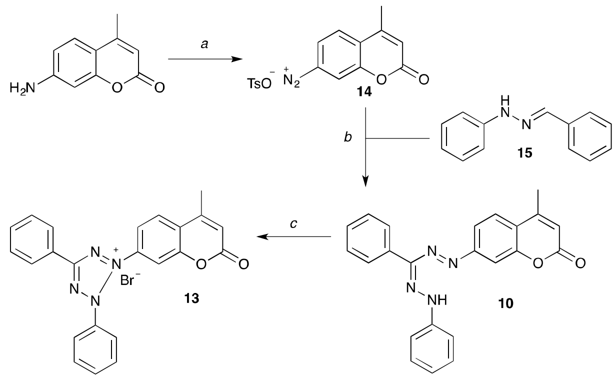
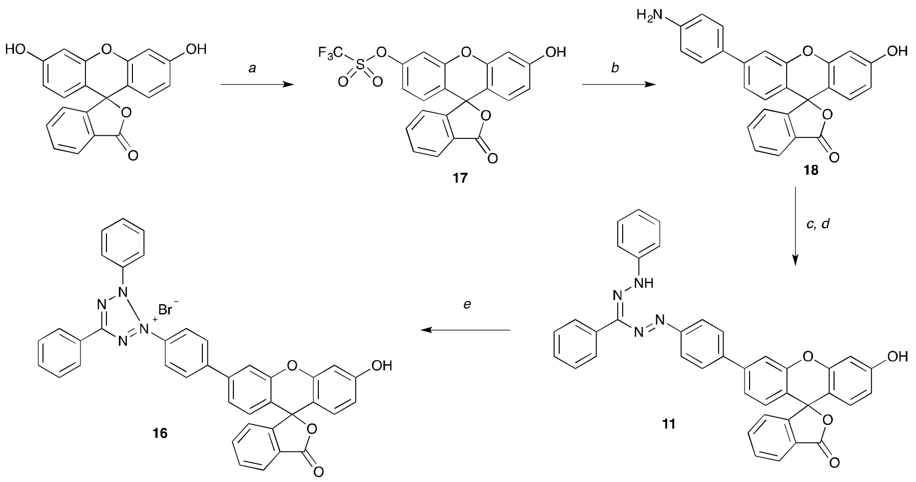
2.1. Synthesis of fluorophore-based formazans and tetrazolium salts

The first target molecule was **10**, which can be considered to be a formazan attached to the fluorophore 7-amino-4-methyl coumarin (AMC), which has an available aniline group for incorporation into the formazan core. Coumarins are of particular interest as they are known to have high quantum yields and retain high fluorescence even following large structural changes [19, 20]. The corresponding coumarin-based tetrazolium salt **13** was synthesised in three steps (Scheme 1). 7-Amino-4-methylcoumarin [20] was diazotised using a solid-supported nitrite resin [21] and **14** was isolated as the *p*-toluenesulfonic acid salt. Benzaldehyde phenylhydrazone **15** [22] was added to the diazonium salt **14** in 2M HCl to give the coumarin–formazan **10** in 62% yield. Oxidition with *N*-bromosuccinimide (NBS) gave the corresponding tetrazolium **13** in 69% yield.



Scheme 1. (a) Amberlyst A26 based nitrite resin, AcOH, *p*-TsOH, 0 °C to rt, 1 h, (64%) (b) 2M HCl, $NaNO_2$, benzaldehyde phenylhydrazone **15**, pyridine, DMF, 4 h (62%); (c) NBS, EtOAc, 50 °C, 20 h (69%).

The "fluorescein-based" tetrazolium salt **16** was synthesised in five steps from fluorescein. Fluorescein monotriflate **17** was synthesised by microwave heating fluorescein and *N*-phenyl-bis(trifluoromethanesulfonimide) ($PhN(OTf)_2$) in the presence of K_2CO_3 [23], followed by a Suzuki coupling with 4-aminophenyl boronic acid pinacol ester under thermal conditions to give **18** in 71% yield (Scheme 2) [24]. Diazotisation of **18** and subsequent treatment with benzaldehyde phenylhydrazone **15** afforded the desired fluorescein–formazan **11** in 29% yield, with oxidation using NBS giving the tetrazolium salt **16** in 86% yield.



Scheme 2. Synthesis of fluorescein–formazan **11** and the corresponding fluorescein–tetrazolium salt **16**. a) $PhN(OTf)_2$, K_2CO_3, DMF, 20 min, mw 80 °C (48%); b) 4-aminophenyl boronic acid pinacolester, $Pd(OAc)_2$, PPh_3, Na_2CO_3, toluene/EtOH (1:1), 80 °C, 22 h (71%), (c) 2M HCl, $NaNO_2$,

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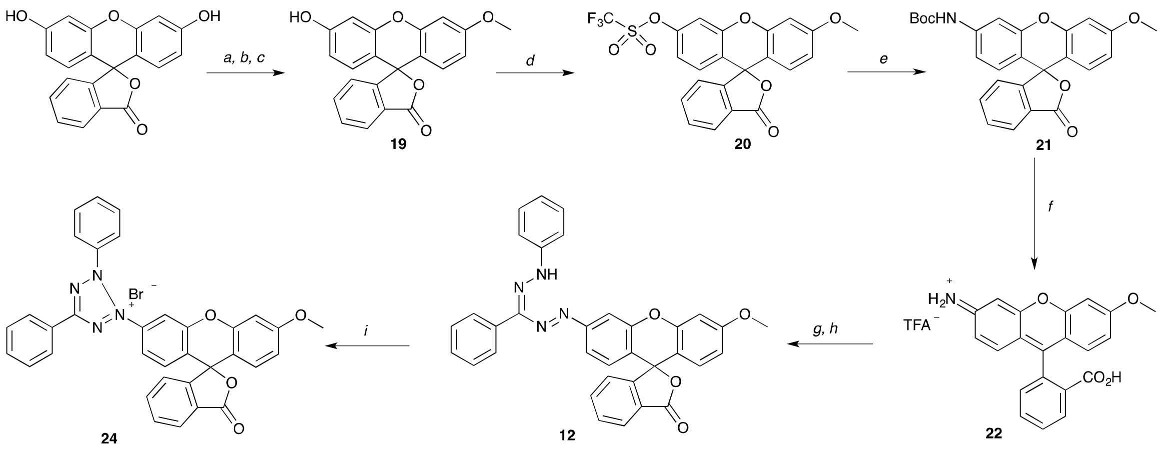
H_2O, 0 °C, 90 min; d) benzaldehyde phenylhydrazone **15**, pyridine, DMF, 0 °C to rt, 18 h (38%); (e) NBS, EtOAc, 50 °C, 24 h (86%).

To allow regioselective incorporation of the formazan moiety into a rhodol-based fluorophore (compound **12**), mono *O*-methyl fluorescein **19** was synthesised [25]. Fluorescein was first treated with methyl iodide (with concomitant methyl ether and methyl ester formation) and subsequent hydrolysis with LiOH gave **19** in 71% yield. Conversion of the phenol moiety in **19** to the corresponding aniline **22** was achieved by the method of Grimm [26], *i.e.*, triflation (compound **20**), followed by Buchwald-Hartwig amination with *tert*-butyl carbamate (compound **21**) and deprotection to afford *O*-methyl rhodol **22** as a TFA salt in 56% overall yield over three steps (Scheme 3). *O*-Methyl rhodol **22** was treated with sodium nitrite, to generate the diazonium salt **23**, which was then treated with diphenylhydrazone **15** to furnish the desired formazan **12** in moderate 25% yield (Scheme 3). NBS oxidation gave the corresponding tetrazolium salt **24**, which precipitated from the reaction mixture and was isolated by filtration in 83% yield.



Scheme 3. Synthesis of **12** and the corresponding tetrazolium salt **24**. a) NaOH, MeOH, 2 h; b) MeI, DMF, 24 h; c) LiOH, MeOH, 100 °C, 24 h (71%); d) $PhN(OTf)_2$, DIPEA, DMF, 2 h (78%); e) *t*-butyl carbamate, $Pd(dbba)_3$, xantphos, $CsCO_3$, dioxane, N_2 atm, 18 h (75%); f) 20%TFA in DCM, 2 h (95%); g) $NaNO_2$, HCl, 0 °C, 1 h; h) **15**, pyridine, DMF, 0 °C to rt, 18 h (25%); i) NBS, EtOAc, 50 °C, 16 h (83%).

2.2. Optical properties of the tetrazolium salts and formazans

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The optical properties of the fluorophore-based formazans and their corresponding tetrazolium salts were analysed and compared to the optical properties of the "parent fluorophores" (Table 1). The formazans **10**, **11** and **12** all showed fluorescence emission between 420 and 470 nm (in EtOH) (Figure 3). The emission wavelength of the formazans was not dependent on the emission wavelength of the parent fluorophore.

Table 1.Optical properties of the synthesised formazans and tetrazolium salts, and their "parent" fluorophores (in EtOH unless otherwise stated).

Compound	λ_{Ex} (nm)	λ_{Em} (nm)	
AMC	350	426	0.78
10	370	468	0.11
13	350	432	0.10
13 pH 4[a]	390	432	0.04
18	480	520	0.26
11	370	445	0.19
16	370	434[b]	–[c]
22	428	523	0.74
12	350	428	0.18
24	350	n/f[d]	–

[a] recorded in acidified PBS/MeOH [b] weakly fluorescent, 4.8-fold difference in emission intensity compared to **11** [c] could not be determined [d] non-fluorescent.

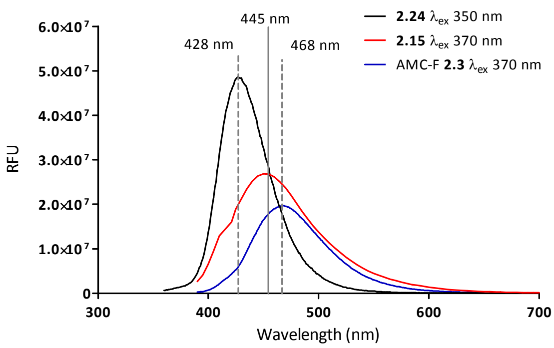

Figure 3. Comparison of fluorescence emission of formazans **10** ($\lambda_{ex/em}$ 370/468 nm), **11** ($\lambda_{ex/em}$ 370/445 nm), and **12** ($\lambda_{ex/em}$ 350/428 nm) in EtOH (recorded at 100 mM).

Coumarin-based formazan **10** showed a 42 nm red shift compared to AMC (ESI, Figure S1), possibly due to the increased conjugation gained from the formazan structure; however, the quantum yield of **10** was lower than that of reported for AMC (Φ 0.11 *versus* 0.78) [27]. The coumarin-based

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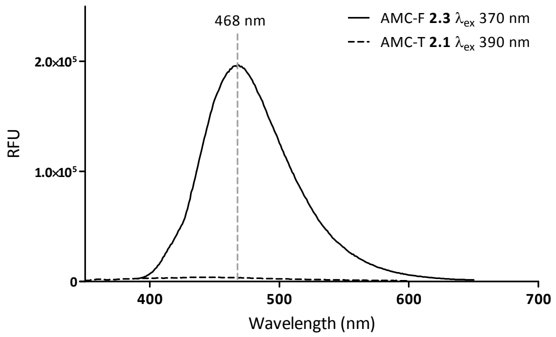
tetrazolium salt **13** remained fluorescent in EtOH (λ_{Em} 432 nm, Φ 0.10) with a 36 nm blue-shift in fluorescence compared to **10** (4-fold difference between the emission intensities (λ_{Ex} 350 nm) of **13** and **10)**. However, under aqueous acidic conditions (1:1 MeOH/PBS with 1% HCl), **13** was almost non-fluorescent whereas formazan **10** retained its fluorescent properties (60-fold difference in emission intensities) (Figure 3A). The difference in fluorescent intensity between **13** and the reduced form **10** could thus be exploited in a cytotoxicity assay where the tetrazolium salt is reduced to a fluorescent formazan and extracted in acidic PBS/MeOH (similar to that used for the MTT assay).

Aniline modified fluorescein **18** had a fluorescence emission maximum at 520 nm (λ_{Em} 480 nm) and a quantum yield of 0.26. When this moiety was incorporated into formazan **11**, the emission was blue-shifted by 75 nm to 445 nm (Φ 0.19) (ESI, Figure S2). The corresponding tetrazolium salt **16** was considerably less fluorescent than the formazan counterpart **11** (xx-fold difference in the emission intensity) (Figure 3B). Similarly with *O*-methyl rhodol-based formazan **12** ($\lambda_{Ex}/\lambda_{Em}$ 370/427 nm, Φ 0.18) the emission was blue-shifted by 96 nm compared to parent fluorohore *O*-methyl rhodol **22** (λ_{Em} 523 nm) (ESI, Figure S3). The tetrazolium salt **24** was non-fluorescent (λ_{Ex} 350 nm) showing that as postulated the positive charge of the tetrazolium salt is able to quench the fluorescence (Figure 3C).



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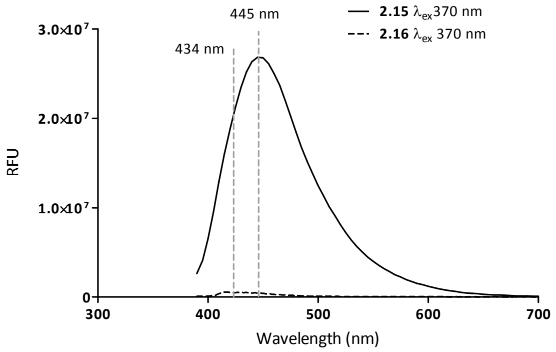


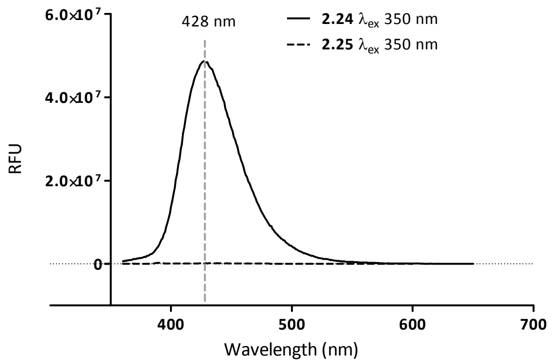
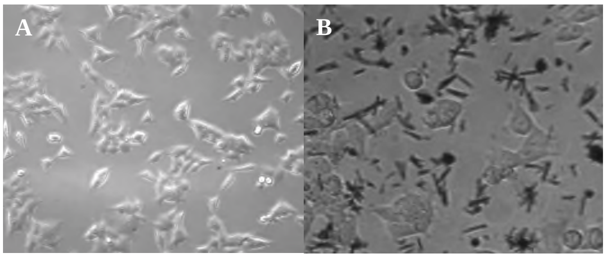


Figure 3. (A) Fluorescence emission of coumarin-based formazan **10** ($\lambda_{ex}/\lambda_{em}$ 370/468 nm) and the corresponding tetrazolium salt **13** (λ_{ex} 390) in acidic PBS–MeOH (1:1). (B) Fluorescence emission of fluorescein–formazan **11** (2.15) emission ($\lambda_{ex/em}$ 370/445 nm) and its tetrazolium salt **16** (2.16) emission ($\lambda_{ex/em}$ 370/434 nm in EtOH; (C) Fluorescence emission of *O*-Methylrholdol-based formazan **12** (2.24) emission ($\lambda_{ex\backslash em}$ 350/428 nm) compared to its tetrazolium salt **24** (2.25) (λ_{ex} 350 nm) recorded in EtOH. All compounds were measured at 100 mM.

3. Cell-based fluorescent analysis

Tetrazolium salts **13**, **16** and **24** (50 μM) were incubated with HeLa cells for 20 hours to allow the reduction to the formazans **10**, **11** and **12**, respectively. Formation of non-soluble crystals was observed on the surface of the cells (Figure 4A–B). The formazan crystals were isolated, solubilised in EtOH, and the fluorescence was measured with the expected emission observed with all the samples (Figure 4C, ESI, Figure S4). For the coumarin-based tetrazolium salt **13** the cell-based reduction into **10** was also confirmed by mass spectrometry of both the supernatant and cell lysate, which confirmed that **13** had been taken up by the cells. Coumarin-based **10** and **13** showed notably cytotoxicity in a MTT assay at 100 μM, whereas with formazans **11** and **12** were non-cytotoxic up to 10 μM (tetrazolium salts **16** and **24** were not toxic at 50 μM) (ESI Figure S5).



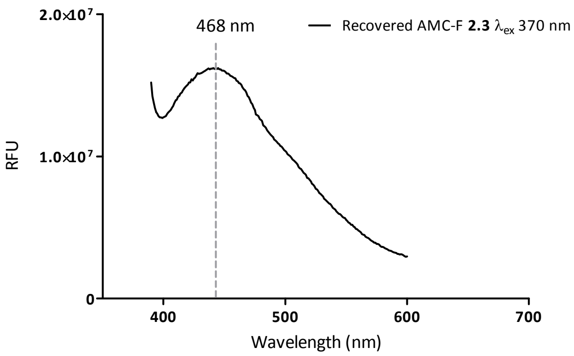


Figure 4. A) HeLa cells immediately after the addition of tetrazolium salt **10** (10 μM). B) Cells following 28 hours incubation showing black formazan **13** crystals (× 100 magnification). C) Recovered AMC-F 2.3; HeLa cells, AMC-T 2.1, Fluorescent emission of recovered **10** after 24 hour incubation of **13** (50 μM) with HeLa. Supernatant was removed and the formazan crystals were collected by centrifugation and dried. Fluorescence was measured in EtOH.

4. CONCLUSIONS

The fluorescent formazans **10**, **11** and **12**, and their corresponding tetrazolium salts **13**, **16** and **24**, respectively, were synthesised by incorporation of a known fluorophore. The large structural changes to the core structure did not affect the *in vitro* activity of the tetrazolium salts, which were successfully reduced to the fluorescent formazans with cells. The emission wavelengths of the fluorophore conjugated formazans were not directly dependent on the parent fluorophores, *i.e.*, the fluorescence of **10** was red shifted compared to AMC whereas both fluorescein-based **11** and *O*-methyl rhodol-based **12** were blue shifted to the green region and were lower than the emission of **10**. The tetrazolium moiety was able to quench the fluorescence of incorporated fluorescein and *O*-methyl rhodol (compounds **16** and **24**, respectively), whereas with the coumarin-based **13** the fluorescence was only quenched in acidic medium, which is typically used in MTT assay.

ACKNOWLEDGEMENTS

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EXPERIMENTAL

All chemicals were purchased from commercial suppliers and used as received. Microwave assisted heating was done in a Biotage Initiator at 2.45 GHz at a fixed temperature. ^{1}H and ^{13}C NMR spectroscopy were recorded on an automated Bruker AVA400 (400 and 100 MHz, respectively) or Bruker AVA500 (500 and 126 MHz, respectively) spectrometer and the chemical shifts are quoted in relative to the solvent. ESI-MS analysis was recorded on an Agilent 1100 system with mass spectra acquired on a VG platform single Quadrupole MS-electrospray positive (ES)+ or negative (ES)- mode. HRMS were acquired using a Bruker MicroToF II in electropositive (ES)+ or electronegative mode (ES)-, or acquired using a Finnigan MAT 900 XLP high-resolution double-focusing mass spectrometer. FT-IR spectra were recorded on a Bruker Tensor 27 with a golden gate accessory for solid samples. Melting points were measured on a Gallenkampf melting point apparatus.

UV absorbance analysis was conducted on an Agilent 8453 spectrophotometer using 100% solvent as blank. Fluorescence emission was measured using a Jobin Yvon SPEX Fluoro Max fluorometer with Data Max version 2.20 software in EtOH unless otherwise indicated. Quantum yields were calculated by comparison to either fluorescein (in 0.1 M NaOH) or harmaline (0.005 M H_2SO_4) standard. Absorbance and fluorescence measurements for quantum yields were acquired with Synergy plate reader loaded with Gen5 (1.1) software using Costar 96-well, flat-bottomed clear plates. Dyes were dissolved in EtOH and quantum yield was calculated using Equation 1

$$\Phi = \Phi_{ref.} \cdot \frac{I_s}{I_{ref.}} \cdot \frac{A_{ref.}}{A_s} \cdot \frac{\eta_s}{\eta_{ref.}}$$

Equation 1. Calculation of quantum yield (Φ); $\Phi_{ref.}$ fluorescein: 0.92, harmaline: 0.32, I_s= integrated fluorescence of the sample, $I_{ref.}$= integrated fluorescence of the reference, $A_{ref.}$= absorbance of reference, A_s = absorbance of the sample, η_s = refractive index of the sample solvent, $\eta_{ref.}$= refractive index of the reference sample (0.1 M NaOH and 0.005 M Na_2SO_4 η= 1.36, EtOH η= 1.36).

7-Amino-4-methylcoumarin diazonium (14)

7-Amino-4-methyl coumarin (0.2 g, 1.0 mmol) was suspended in AcOH (10 mL) and cooled to 0 °C. Amberlyst A26 based nitrite resin [91] (0.7 g, 3.0 mmol) was added portion-wise over 20 min, followed by *p*-toluenesulfonic acid (0.6 g, 3.0 mmol). The mixture was warmed to room temperature and stirred for 1 h. The mixture was filtered and the resulting amber filtrate was poured into cold diethyl ether (100 mL) and the resulting precipitate was collected and washed with cold ether (30 mL) to give **14** as a beige solid (0.2 g, 64%). mp 135–140°C; IR νmax (cm^{-1}) 2284 (N≡N), 1728 (C=O); ^{1}H NMR (d_6-DMSO, 400 MHz) δ 8.77 (1H, d, *J* = 2.0 Hz), 8.61 (1H, dd, *J* = 8.8, 2.0 Hz), 8.29 (1H, d, *J* = 8.7 Hz), 7.46 (2H, d, *J* = 8.0 Hz), 7.10 (2H, d, *J* = 7.9 Hz), 6.88 (1H, d, *J* =1.4 Hz), 2.51 (3H, s), 2.29 (3H, s); ^{13}C NMR (100 MHz, d_6-DMSO) δ 157.7, 151.7, 151.2, 145.5, 137.5, 129.5, 128.2, 128.0, 127.4, 125.4, 120.4, 120.2, 116.9, 22.7, 17.9; MS (ESI) m/z 381.1 $[M+Na]^+$

3-(7-Amino-4-methylcoumarin)-2,5-phenyl formazan (10)

Benzaldehyde phenylhydrazone **15** (78 mg, 0.4 mmol) in DMF (0.5 mL) and pyridine (0.1 mL), was cooled to 0 °C, and 7-amino-4-methylcoumarin diazonium salt **14** (144 mg, 0.4 mmol) in cold 2M HCl (0.2 mL) was added drop-wise. The mixture was stirred at room temperature for 16 h. Upon completion, the solution was poured into water (5 mL) and conc. HCl was added until the solution was acidic (pH 1). The resulting precipitate was filtered, washed with water (10 mL), and dried *in vacuo*. Purification by column chromatography (50% EtOAc in hexane) gave **10** as a dark purple powder (68 mg, 62%). mp 204–209 °C; IR νmax (cm^{-1}) 3052 (NH), 1721 (C=O), 1607 (N=N), 1507 (C=N), 1219 (CN); ^{1}H NMR (500 MHz, $CDCl_3$) δ 15.00 (1H, s), 8.12 (2H, d, *J* = 7.4 Hz), 7.90 (2H, d, *J* = 7.5 Hz), 7.58−7.55 (3H, m), 7.52−7.46 (4H, m), 7.39 (1H, t, *J* = 7.3 Hz), 7.33 (1H, dd, *J* = 8.6, 2.0 Hz), 6.19 (1H, d, *J* = 1.1 Hz), 2.43 (3H, d, *J* = 1.1 Hz); ^{13}C NMR (126 MHz, $CDCl_3$) δ 161.0, 155.1, 152.2, 151.3, 147.6, 142.1, 136.5, 131.3, 129.6, 128.5, 128.4, 126.4, 125.8, 121.8, 116.3, 113.0, 112.3, 103.1, 18.7; HRMS (ESI) $[M-H]^-$ m/z calculated for $C_{23}H_{17}O_2N_4$ 381.1352, obtained 381.0924; UV/VIS (EtOH) λ_{max} 387 nm; $\lambda_{ex/em}$ 370/468 nm; Φ 0.11.

3-(7-amino-4-methyl coumarin)-2,5-phenyl tetrazolium bromide (13)

Formazan **10** (60 mg, 0.16 mmol) was suspended in EtOAc (0.68 mL) and heated to 50 °C. To this *N*-bromosuccinimide (74 mg, 0.20 mmol) in EtOAc (0.48 mL) was added and the mixture was stirred at 50 °C for 12 hours. After cooling to room temperature, the cream precipitate was collected by filtration and dissolved in DCM (3 mL). The solution was washed with aqueous sodium tetrafluoroborate (5 mL), dried over Na_2SO_4, and evaporated *in vacuo* to give **13** as a white powder (42 mg, 69%). No purification was required. mp 234−239 °C;; IR νmax (cm^{-1}) 1721 (C=O), 1457 (C=N); ^{1}H NMR (400 MHz, d_6-DMSO) δ 8.18 (2H, d, *J* = 7.1 Hz), 8.01 (1H, dd, *J* = 8.6, 2.0 Hz), 7.95 (2H, d, *J* = 7.7 Hz), 7.81 (1H, d, *J* = 2.0 Hz), 7.70 (1H, t, *J* = 8.6 Hz), 7.63 (1H, t, *J* = 7.6 Hz), 7.60–7.54 (5H, m), 6.38 (1H, d, *J* = 1.2 Hz), 2.42 (3H, d, *J* = 1.1 Hz); ^{13}C NMR (126 MHz, d_6-DMSO) δ 161.2, 155.3, 152.4, 151.5, 147.8, 142.4, 136.8, 130.9, 129.2 (2 ×), 128.2 (2 ×), 128.0, 125.9 (2 ×), 125.4, 121.5 (2 ×), 116.6, 112.60, 111.9, 102.7, 18.9; ESI-MS [M]+ m/z 381.1; HRMS (ES) [M]+ m/z calculated for $C_{23}H_{17}O_2N_4^+$ 381.1352 obtained 381.1069; UV/VIS (EtOH) λ_{max} 297 nm; $\lambda_{ex/em}$ 350/432 nm; Φ 0.10 (EtOH) and 0.04 (in acidic PBS/MeOH).

3-(Spiro[isobenzofuran-1(3H),9'-[9H]xanthen]-3-one,3'-hydroxy-6'-(4-amino phenyl)-4,5-phenylformazan (11)

Sodium nitrite (41 mg, 0.58 mmol) was added to **11** (185 mg, 0.45 mmol) in 2M HCl (3 mL) at 0 °C and stirred for 90 minutes. This solution was added drop-wise to a solution of diphenylhydrazone **15** (96 mg, 0.9 mmol) in DMF (1.2 mL) and pyridine (0.6 mL) at 0 °C. The reaction was stirred at room temperature for 22 hours. The reaction mixture was poured into water (10 mL) and acidified with 2M HCl to pH 1, the precipitate was collected by filtration, and dried at 40 °C in a vacuum oven for 24 hours. Purification by column chromatography (30% EtOAc in hexane) gave a **16** as dark red powder (104 mg, 38%). mp171−175 °C; IR νmax (cm^{-1}) 3373 (OH), 3032 (NH), 1728 (C=O); ^{1}H NMR; (500 MHz, DMSO) δ 14.11 (1H, s), 10.10 (1H, s), 8.04 (1H, d, *J* = 7.6 Hz), 7.99 (2H, d, *J* = 7.2 Hz), 7.93−7.88 (6H, m), 7.82 (1H, dt, *J* = 7.5, 1.0 Hz), 7.75 (2H, dd, *J* = 8.1, 4.6 Hz), 7.56−7.53 (2H, m), 7.51−7.47 (3H, m), 7.39 (2H, d, *J* = 7.3 Hz), 7.34 (1H, d, *J* = 7.6 Hz), 6.85 (1H, d, *J* = 8.3 Hz), 6.75 (1H, d, *J* = 2.3 Hz), 6.64 (1H, d, *J* = 8.7 Hz), 6.59 (1H, dd, *J* = 8.7, 2.3 Hz); ^{13}C NMR (126 MHz, d_6-DMSO) δ 169.1, 152.4, 151.7, 151.0, 148.3,146.9, 142.3, 141.9, 136.4, 135.7, 135.6, 130.2, 129.7 (2 ×), 129.4, 128.8, 128.7 (2 ×), 128.6, 128.2 (2 ×), 128.1, 126.7 (2 ×), 125.7, 124.7, 124.0, 122.1, 121.0, 120.0 (2 ×), 119.2 (2 ×), 117.7, 114.3, 112.8, 109.1, 102.2, 91.7; HRMS (ESI) $[M+H]^+$ m/z calculated for $C_{39}H_{27}O_4N_4$ m/z 615.2023 obtained 615.2024; UV/VIS (EtOH) λ_{max} 300, 500 nm; $\lambda_{ex}/_{em}$ 370/445 nm; Φ 0.19.

3-(Spiro[isobenzofuran-1(3H),9'-[9H]xanthen]-3-one,3'-hydroxy-6'-(4-amino phenyl)-4,5-phenyltetrazolium bromide (16)

Fluorescein formazan **16** (11.3 mg, 0.02 mmol) was suspended in EtOAc (0.3 mL) and heated to 50 °C. *N*-bromosuccinimide (10 mg, 0.05 mmol) in EtOAc (0.3 mL) was added, and the mixture stirred at 50 °C for 12 hours. The precipitate was collected by filtration to give **16** (9.7 mg, 86%). No purification was required. mp 171−175 °C; IR νmax (cm^{-1}) 3359 (OH), 3034 (CH), 1758 (C=O), 1596 (C=N); ^{1}H NMR (400 MHz, MeOD) δ 8.39 (2H, d, *J* = 7.1 Hz′), 8.10 (1H, d, *J* = 7.3 Hz), 8.05 (2H, d, *J* = 8.6 Hz), 7.88–7.87 (3H, m), 7.85–7.81 (5H, m), 7.77 (2H, d, *J* = 7.3 Hz), 7.76–7.72 (5H, m), 7.51 (1H, d, *J* = 8.6 Hz), 7.30 (1H, d, J *J* = 8.0 Hz), 6.98 (1H, d, *J* = 8.0 Hz), 6.91 (1H, s); ^{13}C NMR (126 MHz, MeOD) δ 161.3, 160.8, 157.9, 156.5, 151.8, 151.5, 145.9, 145.2, 145.1, 141.0, 139.9, 139.8 (2 ×), 138.6, 138.0, 137.9 (2 ×), 137.8, 137.5, 137.5 (2 ×), 135.9 (2 ×), 134.3, 133.7, 131.7, 129.2 (2 ×), 129.1, 128.5, 128.4 (2 ×), 128.3, 127.3, 123.9, 123.8, 122.2, 118.9, 118.8; ESI-MS m/z 613.3 $[M]^+$; UV/VIS (EtOH) λ_{max} 300 nm; $\lambda_{ex}/_{em}$ 370/432 Φ 0.01.

1-(Methylrhodol)-3,5-diphenylformazan (12)

Sodium nitrite (12 mg, 0.17 mmol) in water (300 µL) was added to *O*-methylrhodol **22** (50 mg, 0.15 mmol) in 2M HCl (0.75 mL) at 0 °C and stirred for 60 minutes. This solution was added to a solution of diphenylhydrazone **15** (30 mg, 0.15 mmol) in cold DMF (1.75 mL) and pyridine (0.35 mL), and the reaction brought to room temperature. The reaction was stirred for 22 hours and then poured into water (10 mL). 2M HCl was added until the solution became acidic (pH 1), the resultant crystals were collected by filtration and washed with water (20 mL). The solid was dried in a vacuum oven at 40 °C to give dark red crystals (20 mg, 25%). No purification required. mp 201–204 °C; IR νmax (cm^{-1}) 3061 (NH), 1760 (C=O), 1614 (C=N), 1505 (NH), 1218 (CN); ^{1}H NMR (500 MHz, $CDCl_3$) δ 15.15 (1H, s,), 8.16 (2H, d, *J* = 7.3 Hz), 8.07 (1H, d, *J* = 7.6 Hz), 7.86 (2H, d, *J* = 7.6 Hz), 7.71 (1H, t, *J* = 6.5), 7.66 (1H, t, *J* = 7.4 Hz ′), 7.59–7.52 (3H, m), 7.51–7.43 (3H, m), 7.41 (1H, t, *J* = 7.3 Hz), 7.22 (1H, d, *J* = 7.5 Hz), 7.12 (1H, dd, *J* = 8.5, 1.9 Hz), 6.86–6.79 (2H, m), 6.73 (1H, d, *J* = 8.8 Hz), 6.65 (1H, dd, *J* = 8.8, 2.4 Hz), 3.85 (3H, s)CH3); ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.5,161.4, 153.2, 152.5, 152.4, 150.5, 147.1, 141.6, 136.9, 135.1, 130.3, 129.8, 129.5 (2 ×), 129.2, 129.0, 128.4 (2 ×), 128.0, 126.6, 126.1 (2 ×), 125.1, 123.9, 121.1 (2 ×), 115.2, 112.7, 111.8, 111.0, 103.8, 100.9, 83.0, 55.6; HRMS (ES) m/z [M+H]+ calculated for $C_{34}H_{25}N_4O_4$ 553.1870 obtained 553.1726; UV/VIS (EtOH) λ_{max} 298 & 483 nm;; $\lambda_{ex/em}$ 350/428 nm; Φ 0.18.

1-Methoxyrhodol-3,5-diphenyl tetrazolium salt (24)

Formazan **12** (20 mg, 0.03 mmol) was suspended in EtOAc (0.6 mL) and warmed to 50 °C. To this *N*-bromosuccinimide (16 mg, 0.09 mmol, 2.5 eq.) in EtOAc (0.4 mL) was added, and the mixture was stirred at 50oC for 16 hours. The precipitate was collected by filtration, to afford a cream powder (15 mg, 83%). No purification was required. mp 142−147 °C; IR νmax (cm^{-1}) 1695 (C=O), 1102 (COC); 1H NMR (500 MHz, CDCl3) δ 8.17 (2H, d, *J* = 7.4 Hz), 8.04 (1H, d, *J* = 7.6 Hz), 7.89 (1H, d, *J* = 2.2 Hz), 7.82 (2H, d, *J* = 7.9 Hz), 7.75 (1H, t, *J* = 7.4 Hz) 7.67−7.64 (2H, m), 7.61–7.49 (5H, m), 7.33 (1H, dd, *J* = 8.6, 2.1 Hz), 7.18 (1H, d, *J* = 7.6 Hz), 6.92 (1H, d, *J* = 8.6 Hz, H8), 6.79 (1H, d, *J* = 1.9

Hz), 6.74–6.66 (2H, m), 3.85 (3H, d, J = 7.2 Hz); ^{13}C NMR (126 MHz, $CDCl_3$) δ 176.9, 168.8, 166.0, 161.8, 152.1, 151.7, 151.6, 135.8, 134.1, 134.0, 133.2, 133.0, 130.5 (2 ×), 130.1, 129.4 (2 ×), 128.8, 128.0 (2 ×), 126.2 (2 ×), 125.8, 125.4, 124.9, 124.0, 122.9, 120.4, 116.0, 113.1, 109.8, 101.0, 81.1, 55.7; HRMS (ES) m/z $[M]^+$ calculated for $C_{34}H_{23}N_4O_4^+$ 555.1714 obtained 555.3324.

Reduction of tetrazolium salt in cells □

HEK293T cells were cultured in T75 culture flasks in DMEM media, supplemented with 10 % FBS, 4 mM glutamine, and 100 units/mL of penicillin/streptomycin, and grown to 50–60% confluence. Tetrazolium salts **13**, **16**, or **24** (0.5 mM in 1 mL DMSO) were added to phenol red free DMEM media (10 mL) to give a final concentration of 50 µM. DMEM cell media was removed and replaced with DMEM with tetrazolium salts and the cells were incubated at 37 °C for 24 hours. The media was removed and the cells washed with PBS three times. The PBS and media were combined and centrifuged (1200 rpm) for 15 minutes. The supernatant was removed leaving the formazan residue, which was dried at 40 °C in a vacuum oven, and then dissolved in EtOH (5 mL) and fluorescence was recorded. The samples were also analysed by mass spectrometry to confirm presence of the formazans **10**–**12**.

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